Metabolism and cell wall incorporation of phenoxyacetic acid in soybean cell suspension culture

François M G Laurent* & René Scalla

Institut National de la Recherche Agronomique, Laboratoire des Xénobiotiques, 180 Chemin de Tournefeuille, BP 3, 31931 Toulouse Cedex 9, France

Abstract: The metabolism of [^{14}C]phenoxyacetic acid (POA) was studied in cell suspension culture of soybean (*Glycine max*). POA was metabolized to 4-HO-POA, 4-HO-POA glucoside and 4-HO-POA glycosidic ester. A large part of the 4-HO-POA glucoside and small amounts of the glycosidic ester were recovered in the medium. POA was also converted to non-extractable residues bound to cell walls. Sequential extraction of cell-wall polymers showed that non-extractable residues, partly identified with 4-HO-POA and POA, were mainly associated with hemicelluloses and lignin. Comparison of the metabolism of [*carboxy*-^{14}C]- and [*phenyl*-^{14}C]POA revealed some degradation of the POA side-chain, followed in all probability by the incorporation of the aromatic moiety into cell walls. However, the sturdiness of the resulting bonds prevented precise identification of these bound aromatic structures. In summary, the degradation of POA in soybean cell culture provided a good model to study the formation of non-extractable residues of pesticides.

Keywords: phenoxyacetic acid; metabolism; soybean; cell culture; non-extractable residues; xenobiotics; plant

1 INTRODUCTION

The degradation of pesticides in higher plants results in the formation of variable amounts of non-extractable, or bound residues. These residues are associated with plant insoluble components, especially cell walls, and their mode of formation is still not thoroughly understood.[1–3] The formation of bound residues from phenoxyalkanoic acids such as 2,4-D has been demonstrated on several occasions.[4–6] Herbicides of this family are widely used and studies of their metabolism have involved whole plants and in-vitro cell cultures.[7–9] Cell suspension cultures offer several advantages. They are sterile, and their metabolism is not influenced by micro-organisms.[10,11] Use of cell cultures obviates the difficulties of penetration and translocation of pesticides in whole plants.[10,11] Uptake of pesticides by cells is uniform, and consequently the interpretation of metabolic studies is quite straightforward.[12] In general, metabolic pathways and terminal metabolites are the same as those described for whole plants.[13,14] Metabolites can be produced in high amounts, which facilitates their extraction and purification.[10,15]

However, comparatively low levels of bound residues are formed in cell cultures, particularly from dicotyledonous plants,[16] and this can make their analysis somewhat difficult. In order to study this, the amount of the applied pesticide can be increased, but that amount is limited by the phytotoxicity of the molecule. To circumvent that difficulty, one can apply a primary metabolite, generally less phytotoxic than the pesticide itself.[17] Another way is to use a nonphytotoxic analogue of the pesticide. We have chosen the latter method in the present work, by using phenoxyacetic acid (POA) as a model of the phenoxyalkanoic acid herbicide family. Earlier studies have shown that, in plant tissues, POA is hydroxylated and converted into glucoside and non-extractable residues.[18–21]

In this paper, we describe the metabolic degradation of POA and the formation of bound residues in a soybean cell suspension culture. The association of the bound residues with the various cell wall polymers is also examined, because it conditions the bioavailability and ecotoxicity of the bound residues.[15]

2 MATERIALS AND METHODS

2.1 Chemicals

Unless otherwise stated, chemicals and enzymes were purchased from Sigma (Saint Quentin Fallavier, France). Radiolabelled POA was synthesized by

* Correspondence to: FMG Laurent, Institut National de la Recherche Agronomique, Laboratoire des Xénobiotiques, 180 Chemin de Tournefeuille, BP 3, 31931 Toulouse Cedex 9, France
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reacting bromoacetic acid with phenol;[22] [*carboxy*-^{14}C]POA was prepared from [1-^{14}C]bromoacetic acid (Amersham), and [*phenyl*-^{14}C]POA from [*U*-^{14}C]phenol (Sigma). Both products were purified by TLC on silica-coated plates (60 F_{254}, 2 mm, Merck). The solvent system was petroleum ether + diethyl ether (40 + 70 by volume). The purified products were dissolved in acetone, and the desired specific radioactivity was obtained by addition of non-radioactive POA.

2.2 Cell cultures and treatments

Cell suspension cultures of soybean (*Glycine max* Merr, cv Weber) were grown in the dark at 25°C in 10 ml of Gamborg B5 medium (pH 5.5) supplemented with 4.5 μM 2,4-D. Cells were transferred every seventh day into fresh medium to maintain the culture in exponential growth.

For metabolic studies, 40 μg of radioactive POA (400 000 dpm) dissolved in acetone (10 μl) were added to the cultures seven days after subculturing. The amount of cell material averaged 1.04 g fresh weight, or 43 mg dry weight. The experiments were performed in four replicates.

2.3 Cell fractionation

Cells were separated from the medium by vacuum filtration on multigrade GMF 150 filter, 2 μm porosity (Whatman), and washed with 25 ml distilled water. They were homogenized in a glass Potter tissue grinder with 40 ml of methanol + dichloromethane + water (2 + 1 + 0.8 by volume), stored overnight at −20 °C then homogenized again with an Ultraturrax mixer, and sonicated for 10 min. The cell debris was removed by vacuum filtration and washed with methanol + dichloromethane + water, then with ethanol, and finally with acetone. The cell debris, which containing mainly cell walls, was air-dried for 24 h, ground with a ball grinder, before recovering the ^{14}C by combustion in a sample oxidizer.

The organic portion of cell extracts was evaporated *in vacuo*, and residual water phases and media were then lyophilised. Preliminary trials showed that all radioactive materials, including those extracted with organic mixtures, were water-soluble. In consequence, the residues were solubilized with water, acidified to pH 3 and directly analysed by HPLC.

2.4 Identification of soluble metabolites

2.4.1 Hydrolysis procedures

All reactions were carried out on previously dried samples containing at least 4000 dpm of the compound under investigation, according to Schmitt *et al.*[23]

Acid hydrolysis. The sample was dissolved in hydrochloric acid (2 M; 200 μl) and heated to 100°C for 2 h.

Alkaline hydrolysis. The sample was incubated with sodium hydroxide (0.1 M; 200 μl) for 1 h at 50°C.

β-Glucosidase hydrolysis. The sample was incubated during 2 h at 30°C with almond β-glucosidase (0.06 U; Sigma G-0395) in sodium acetate solution (0.1 M, pH 5.0; 200 μl).

Esterase hydrolysis. The sample was incubated (10 min, 30°C) with potassium phosphate (0.05 M, pH 7.5; 200 μl), containing rabbit liver esterase (2.5 U; Sigma E-9636). After hydrolysis, alkaline samples were acidified to pH 3 with hydrochloric acid. Acid and alkaline hydrolysates were extracted three times with ether and analysed by HPLC. Enzymatic hydrolysates were acidified with hydrochloric acid and directly analysed by HPLC.

2.4.2 Reference compounds

4-Hydroxy-POA (4-HO-POA) was synthesized by mole to mole reaction of hydroquinone with bromoacetic acid in 0.1 M sodium hydroxide under nitrogen. The product was purified by thin-layer chromatography. The 4-HO-POA glucoside was prepared by the Koenigs–Knorr reaction.[24] The 4-HO-POA glucose ester was synthesized from the 4-hydroxyphenoxyalkanoyl chloride by the reaction described by Plusquellec *et al.*[25] The glucosides were purified by HPLC as described below (see Section 2.6).

2.5 CW fractionation

CW were Soxhlet-extracted with ethanol + benzene (9 + 1 by volume) for 48 h to give the free cell wall fraction (CW), which was stored under nitrogen in a desiccator until used. CW polymers were extracted from samples weighing about 20–50 mg, with a sequence of chemical and enzymatic procedures as detailed in Table 1.

All extractions were performed with continuous shaking. Chemical extractions were performed under nitrogen. Enzymatic digestions were carried out in reaction mixtures containing 0.1 M buffers with 0.5 g litre^{-1} chlorobutanol. For cellulose extraction, Cellulysin (Calbiochem) was tested to be free of esterase activity according to Ralph *et al.*[26] Release of glucose was monitored with the Sigma Glucose (HK) assay kit, and cellulose hydrolysis was pursued until no more glucose was released.

After each extraction step, insoluble residues were collected by centrifuging (10 000*g* for 10 min) and washed with the buffer used for enzymatic digestion, then with water, or for chemical steps, directly with water. The wash solutions were combined with the respective extraction solutions, and an aliquot of the combined extract was taken to determine the extracted radioactivity.

	Polymer	*Extraction procedure*	*Amount of polymer* (%)[a]
1	Water-soluble polysaccharides	Water, 1 h at 4°C, then 1 h at 100°C	13.3[b]
2	Acidic pectins	EDTA (5 g litre^{-1}) 1 h at 100°C	11.2[b]
3	Proteins	Pronase (0.5 g litre^{-1}; pH 7.5) 24 h at 25°C	nd[c]
4	Cellulose	Cellulysin (10 g litre^{-1}; pH 5.0) 24 h at 25°C, repeated six times	8.8[b]
5	Björkman lignin	Dioxane + water (8 + 2 by volume) 96 h at room temperature	2.6[d]
6	WE-LCC[e]	Water 24 h at 20°C, then 5 h at 80°C	nd[c]
7	Hemicelluloses	Trifluoracetic acid (2 M) 1 h at 100°C, repeated four times	16.5[b]
9	Klason lignin	Residuum	2.0[d]

[a] Determined on cell wall sample of *c*.20 mg.
[b] Based on carbohydrate determination, as described by Fry.[36]
[c] nd = not determined.
[d] Estimated by the acetyl bromide/acetic acid method.[36]
[e] Water-extractable lignin-carbohydrate complex.

Table 1. Sequential extraction of CW polymers

2.6 Analytical procedures

2.6.1 Chromatography

The extracts were analysed by reverse-phase HPLC with a Spectra-Physics system consisting of a P4000 pump, a Rheodyne 7125 injection valve with a 200 µl injection loop, and a model P1000 Spectra-Physics UV detector set at 216 nm. Separations were carried out on a C18, 6-µm Bischoff column with a guard cartridge. Elutions were performed at ambient temperature at a flow rate of 1 ml min^{-1} with acetonitrile (solvent A) and 2 ml litre^{-1} acetic acid in distilled water (solvent B) as mobile phases. Elution gradient was as follows: 10% A to 50% A from 0 to 25 min, then 50% A from 25 to 30 min.

POA and its metabolites were compared to the synthesized standards on the basis of their retention times: POA, 20.8 min; 4-HO-POA, 7.50 min; 4-HO-POA glucoside, 2.50 min; 4-OH-POA glucose ester, 4.00 min.

2.6.2 Radioanalysis

All counting cocktails and materials were from Canberra-Packard. ^{14}C-labelled samples were dissolved in Ultima Gold cocktail. The radioactivities were measured with a Packard C2400 liquid scintillation counter. The radioactivities of insoluble residues were recovered by combustion with an oxidizer, and counted in Carbosorb cocktail. The radioactivity of column effluents was monitored with an on-line Flow-One\beta scintillation detector and Flow-Scint II cocktail, or in fraction aliquots with the liquid scintillation counter.

3 RESULTS

3.1 Uptake of [*carboxy*-^{14}C]POA and distribution of the radioactivity

Figure 1 shows the distribution of radioactivity after application of [*carboxy*-^{14}C]POA to a cell culture. After 10 h of incubation, 77.6% of the radioactivity was associated with the cells. Most of that radioactivity (87.6%) could be extracted by a methanol-dichloromethane-water mixture. The rest was bound to insoluble cell materials, and these non-extractable residues represented 9.6% of the total applied radioactivity. In heat-inactivated cultures (100°C for 15 min), only 2% of the applied radioactivity was associated with non-extractable residues after 72 h of incubation. Therefore, the radioactivity of that fraction represented genuine POA metabolites.

The radioactivity in culture medium remained at a relatively stable level, and accounted for between 15 and 19% of the total. By contrast, the radioactivity of cellular extracts continuously decreased during the incubation, down to 34.2% of the total at 72 h. The radioactivity of the non-extractable fraction increased up to 26.3% at 48 h, and did not significantly change afterwards. At 72 h, non-extractable residues represented 27.4% of the total radioactivity, and 44.5% of the cell-associated radioactivity.

At all incubation times, a certain proportion of the applied radioactivity was not recovered in any fraction of the cell culture. That loss was rather limited

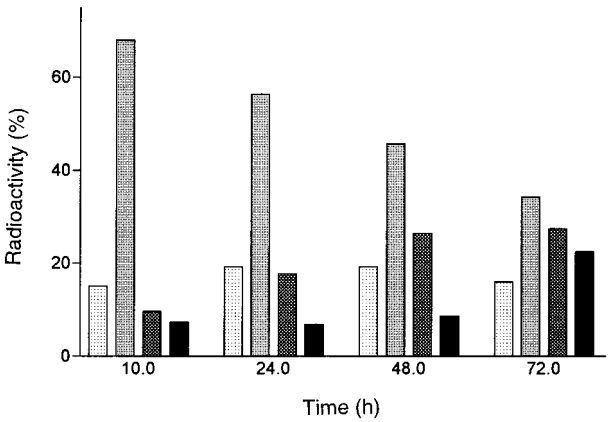

Figure 1. Uptake and distribution of [*carboxy*-^{14}C]POA in soybean cell culture. Results are expressed as percentages of the total radioactivity. (▨) Medium; (▩) cellular extract; (▧) unextractable residues; (■) calculated loss.

up to 48 h, but it reached 22.3% after 72 h of incubation. Accordingly, the decrease of the radioactivity of the cellular extracts was compensated by a simultaneous increase of the non-extractable radioactivity until 48 h of incubation, but at 72 h, the decrease resulted from a loss of radioactivity carbon dioxide as will be shown below.

3.2 Soluble metabolites of [*carboxy*-^{14}C]POA

HPLC analysis indicated a fast metabolism of POA, 83.9% of which had disappeared after 10 h of incubation (Fig 2 C). POA was essentially found in the culture medium (Fig 2 A). Its very low cellular concentration (Fig 2B) showed that it was metabolized immediately upon its entry into the cells. In heat-inactivated cultures, a slow disappearance of POA was detected (6.7($\pm$ 1.5)% at 48 h), but without any accompanying metabolite formation.

Besides POA, three major metabolites were detected in cultures: 4-HO-POA, 4-HO-POA glucoside, and 4-HO-POA glyco-ester conjugate (Fig 3). Figure 2C shows the kinetics of the metabolization in the whole culture. At 10 h, the major compound was identified as the hydroxylated metabolite 4-HO-POA, which represented 76.3% of the soluble metabolites. Thereafter, its amount decreased to 36.4% of the total soluble metabolites (ie 15.6% of the applied radioactivity). This primary metabolite was abundant in cells, but it was absent from culture media (Figs 2 A and B).

The two other main metabolites of cellular extracts were identified as glyco-conjugates of 4-HO-POA. Peak I co-chromatographed with 4-HO-POA glucoside. This identity was confirmed by β-glucosidase treatment, which released the 4-HO-POA aglycon (84% recovery). Peak II co-chromatographed with authentic 4-HO-POA glucose ester. This compound was resistant to β-glucosidase, but was hydrolysed by 0.1 M NaOH or liver esterase. The latter treatment released 4-HO-POA with a 68% yield. The amount of both glycoconjugates increased during the experiment (Fig 2C) and reached 50.5% of the total soluble metabolites (22.0% and 28.5%, respectively for 4-HO-POA glucoside and 4-HO-POA glyco-ester conjugate).

Minor amounts of other metabolites were occasionally detected in cellular extracts, but they were not formally identified. One of them (Fig 3B, Rt 6.40 min) was probably the malonyl-ester derivative of 4-HO-POA glucoside, as suggested by the following observations: the compound was relatively unstable during its purification; it was completely resistant to β-glucosidase, but was cleaved by weak alkaline or esterase treatment, which liberated 4-HO-POA glucoside. Other peaks, poorly separated from POA, never accounted for more than a few percent of the soluble radioactivity.

The distribution of 4-HO-POA conjugates between culture medium and cells is shown in Figs 2A and 2B, and Fig 3. Both conjugates were found in the medium. However, 4-HO-POA glucoside was present in all samples, whereas the glyco-ester conjugate was not always detectable (Fig 3A), and accounted for less than 1% of the applied radioactivity (and from 0 to 7.3% of the radioactivity of the medium). The metabolites were unequally distributed between the medium and the cells: the 4-HO-POA glucoside was approximately two times more

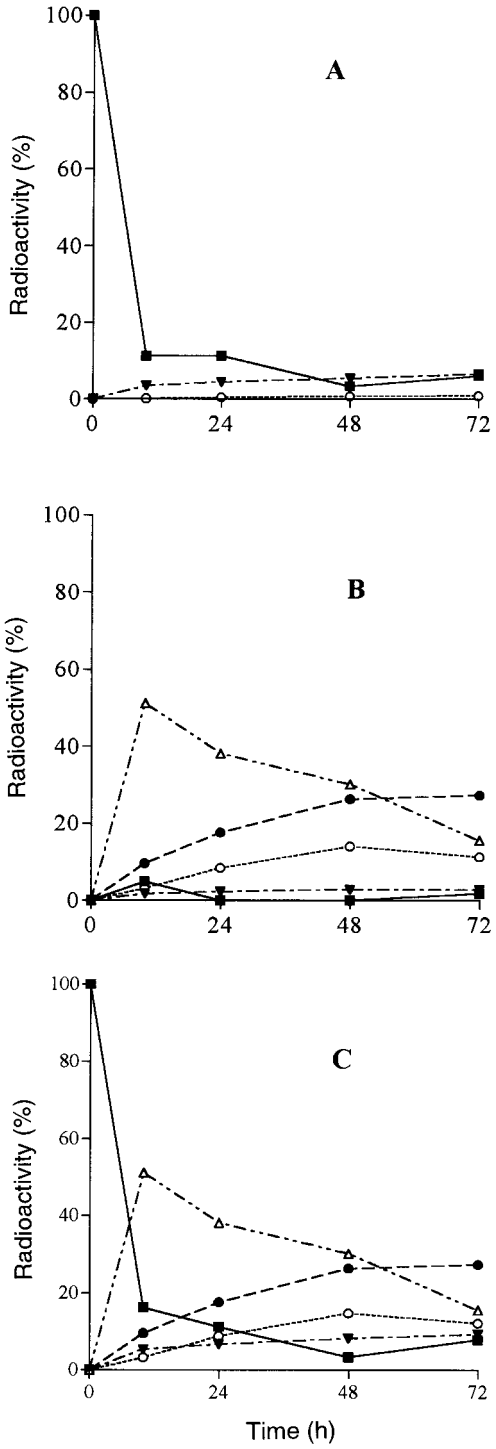

Figure 2. Time course of [*carboxy*-^{14}C]POA metabolism in soybean cell culture. Results are expressed as percentages of the total radioactivity. (A) Culture medium, (B) cells, (C) total culture. (■) POA; (△) 4-HO-POA; (▼) 4-HO-POA-glucoside; (○) 4-HO-POA-glyco-ester; (●) unextractable residues.

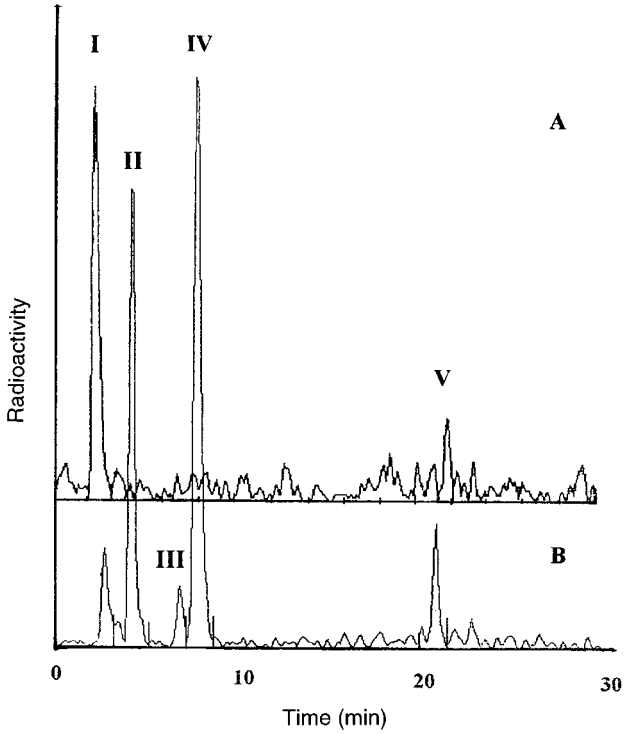

Figure 3. HPLC analysis of soluble metabolites in (A) culture medium and (B) cellular extract 72 h after treatment by [*carboxy*-^{14}C]POA. I: 4-HO-POA-glucoside; II: 4-HO-POA-glyco-ester; III: unidentified metabolite; IV: 4-HO-POA; V: POA.

abundant in the medium; by contrast, the cells contained 12 times more 4-HO-POA glyco-ester conjugate than the medium. These differences showed that the presence of metabolites in the medium was not due to cell lysis and the ensuing release of cell components.

3.3 Comparative metabolism of [*carboxy*-^{14}C]POA and [*phenyl*-^{14}C]POA

As shown above, a sizeable loss of radioactivity was observed during the degradation of [*carboxy*-^{14}C]POA. This deficit was particularly pronounced at 72 h of incubation, where it amounted to 22% of the applied radioactivity. Figure 1 shows that the loss markedly increased between 48 and 72 h of incubation. During that period, the radioactivity of the cellular extract decreased, but that of the non-extractable fraction remained stable, which suggests that some degradation occurred.

The origin of the deficit was then examined, and a preliminary experiment with a larger sample (100 ml culture) showed that when the carbon dioxide produced by the culture was trapped in barium hydroxide solution, the collected radioactivity amounted to 15% after 48 h, with a total recovery of 96.5%. Since this radioactivity originated from the carboxyl group of POA and suggested a degradation of the side chain, further metabolic studies were performed with POA labelled on the phenolic ring. In that second series of experiments, the metabolism of [*carboxy*-^{14}C]POA and [*phenyl*-^{14}C]POA were followed in parallel. After 72 h of incubation, 11.4% of additional radioactivity was recovery in the culture supplied with [*phenyl*-^{14}C]POA (Table 2), confirming that the evolved carbon dioxide originated from the POA carboxyl group. The radioactivities of the culture medium and the cellular extracts were not significantly different in the two experimental systems (Table 2). Moreover, the application of [*phenyl*-^{14}C]POA did not reveal any new metabolite by comparison with [*carboxy*-^{14}C]POA. By contrast, however, incubation with [*phenyl*-^{14}C]POA resulted in 26.6% of the radioactivity incorporated in non-extractable residues, vs 14.8% with [*carboxy*-^{14}C]POA, which corresponded to an 80% increase.

3.4 Cell wall incorporation

The above results showed a significant incorporation of POA metabolites into insoluble cell components, mainly originating from cell walls. To determine the fixation sites of the non-extractable residues, the cell wall from cultures supplied with [*carboxy*-^{14}C]- or [*phenyl*-^{14}C]POA were isolated and fractionated into their macromolecular components (Table 3).

The experiments were done with parallel subcultures at the same cell density. The amounts of the

Table 2. Distribution of radioactivity in soybean cell cultures, 72 h after application of radioactive POA

Fraction	*Percentage of applied radioactivity* ($\pm$ *SD*)[a]	
	[*carboxy*-^{14}C]POA	[*phenyl*-^{14}C]POA
Culture medium	8.2 ($\pm$1.6)	10.5 ($\pm$0.7)
Cellular extract	60.3 ($\pm$1.4)	58.2 ($\pm$2.2)
Non-extractable residues	14.8 ($\pm$1.0)	26.6 ($\pm$1.9)
Total recovered radioactivity	83.2 ($\pm$1.3)	94.6 ($\pm$2.6)

[a] Four parallel experiments.

Table 3. Bound-residue distribution in cell-wall polymers. Cell suspension cultures of soybean were incubated for 72 h with [*carboxy*-^{14}C]POA or [*phenyl*-^{14}C]POA

Polymer fraction	[*carboxy*-^{14}C]POA		[*phenyl*-^{14}C]POA	
	Radioactivity (*dpm*)[a]	(%)[b]	*Radioactivity* (*dpm*)[a]	(%)[b]
Pectins	84	10.7	46	3.4
Starch	—	trace	—	trace
Proteins	25	3.2	6	0.4
Cellulose	39	4.9	19	1.4
Björkman lignin	13	1.6	24	1.8
WE-LCC[c]	7	0.9	–	trace
Hemicellulose	273	34.6	445	33.2
Residuum (Klason lignin)	353	44.8	799	59.5
Total	788	100.0	1342	100.0

[a] Radioactivity incorporated in 1 mg of cell walls.
[b] Percentage of the total cell wall radioactivity.
[c] WE-LCC: Water-extractable lignin-carbohydrate complex.

cell wall fractions were similar in the two samples, with average weights of 60.7 and 63.3 mg cell wall per sample for [*carboxy*-^{14}C]- and [*phenyl*-^{14}C] POA, respectively. Incorporation of radioactivity from [*phenyl*-^{14}C]POA amounted to 1342 dpm mg^{-1} cell wall, vs 788 dpm from [*carboxy*-^{14}C]POA. Incubation of [*phenyl*-^{14}C]POA thus resulted in 70% more incorporation in the cell walls.

In both cell wall batches, radioactivity was mainly bound to hemicelluloses and lignins (Table 3). On a cell wall weight basis, the radioactivity incorporated in these polymers was clearly higher with [*phenyl*-^{14}C]POA (63 and 126% increase for hemicelluloses and lignin, respectively). Relatively small amounts of residues were associated with other polymers. Some data were surprising at first sight: cellulose, pectins and proteins incorporated more radioactivity from [*carboxy*-^{14}C]POA than from [*phenyl*-^{14}C]POA (Table 3).

Alkaline hydrolysis (6 M NaOH, 170°C, 2 h) of cell walls labelled with both types of precursor released more than 90% of bound radioactivity. However, HPLC analyses of hydrolysates showed that, whatever the POA label position, the main identified residue was 4-HO-POA, with some parent POA. Obviously, the difficulties of recovery and analysis of released residues did not allow acquisition of reliable quantitative data, particularly concerning the metabolites resulting from the dealkylation of POA ([*phenyl*-^{14}C]POA samples).

4 DISCUSSION

In the present work, the metabolism of POA and the incorporation of its residues into cell walls has been studied in soybean cell cultures. Owing to its low phytotoxicity, POA can be applied at a much higher concentration than 2,4-D (263 μM vs 9 μM, F Laurent, unpublished observations). Another interest of POA is the relative simplicity of its metabolic pathway in plants,[5,19–21] by comparison with 2,4-D.[6]

Analyses of POA metabolites in cell cultures confirmed that simplicity, since they revealed only one primary metabolite, 4-HO-POA, and two glycoconjugates: the 4-HO-POA glucoside and the 4-HO-POA glyco-ester conjugate. Surprisingly, the glyco-ester of POA was not detected. On the other hand, incubation with [*phenyl*-^{14}C]POA revealed a decarboxylation pathway, but no corresponding metabolite was detected. A similar pathway has been demonstrated for some phenoxyalkanoic acids.[27] Its activity is negligible for 2,4-D in soybean culture,[9] but significant for higher isomers.[27]

The first object of this study was to set up a cell culture system able to metabolize a high proportion of POA derivatives into non-extractable residues. That requirement was met, since for example after 48 h incubation with [*phenyl*-^{14}C]POA, more than 25% of the radioactivity was incorporated into cell-wall polymers. The rates of incorporation into non-extractable residues were substantially higher than those described for 2,4-D, which range from 2 to 10% in soybean cultures.[9,12,28] The apparent rate of incorporation depended, however, on the position of the label on the parent molecule. In the case of [*carboxy*-^{14}C]POA, carbon dioxide evolution was indicative of loss of the carboxylic carbon. The higher apparent incorporation of [*phenyl*-^{14}C]POA showed that decarboxylated, or more likely dealkylated metabolites, were totally incorporated into cell walls. However, a complete demonstration of that incorporation could not be achieved. Although an alkaline hydrolysis released all the bound radioactivity, only 4-HO-POA and POA could be identified in the hydrolysate. On the other hand, other experiments have shown that phenolic compounds (particularly hydroquinone) can be detected in the cell walls of soybean plants after absorption of [*phenyl*-^{14}C]POA (F Laurent, unpublished results).

In order to determine the distribution of residues in cell walls, a protocol allowing selective extraction of the various cell wall polymers without loss of their respective bound residues was used. In the present study, whatever the position of the label on the POA molecule, residues were mainly bound to lignin and hemicelluloses. Moreover the comparison of results obtained with [*carboxy*-^{14}C]- and [*phenyl*-^{14}C]POA opens the possibility that decarboxylated metabolites were incorporated in these two polymers, although cell-wall analyses did not clarify this point. By contrast, the comparatively higher incorporation of [*carboxy*-^{14}C]POA residues in pectins, proteins and cellulose can be explained by the incorporation into plant constituents of carbon atoms released by the dealkylation reaction after their oxidation to carbon dioxide.[29] Therefore, the use of POA labelled on the carboxylic function leads to overestimation of the non-extractable residues associated with these polymers.

The distribution of POA residues in the cell walls of soybean cell cultures was similar to that observed for leaves of soybean plants (F Laurent, unpublished results). These cultures are thus good models for the study of non-extractable residue formation. However, it must be kept in mind that, whatever the advantages of that system, it does not exactly reflect the metabolism of the whole plant. Indeed, the distribution of bound POA residues in soybean stems seems different, with more residues bound to pectins than to hemicelluloses (F Laurent, unpublished results). That characteristic is probably related to the abundance in stems of cells surrounded by secondary walls and equipped with relevant metabolic pathways.[30] By contrast, secondary walls are largely absent from soybean cell cultures.

The incorporation of xenobiotic residues in cell-wall macromolecules is often considered as a local excretion pathway, in parallel to the vacuolar compartmentation.[3,31] Some xenobiotics are incorporated into cell walls as primary metabolites,[32] and

Hutber *et al*[19] have shown that 4-HO-POA can be the direct precursor of the POA bound residues in pea. That also could to be the case for dealkylated POA metabolites, since no glucoside of these compounds was detected. This last possibility, however, still has to be confirmed by direct experimental evidence. On the other hand, vacuolar uptake is preceded by the formation of glucosyl or malonyl-glucosyl conjugates.[23,33] By analogy, some authors consider glucoside or malonyl-glucoside conjugates to be the precursors of bound residues,[32] which are incorporated into cell walls after deglucosylation. Other authors[34] describe the vacuolar deposition as an intermediate step between the secondary metabolism and the formation of bound residues; vacuolar glucosides would be transported in the cytosolic and the apoplastic media, before being hydrolysed and incorporated into cell walls.[3] In our soybean cell cultures, the glucoside of 4-HO-POA was the only secondary metabolite present in large amounts in the extracellular medium. However, it is not possible to decide if that extracellular glucoside was a precursor of bound residues, or if it was simply excreted in the apoplastic space as a toxic waste.

The study of non-extractable residue information in plants should provide some information concerning the bioavailability of these compounds in the environment and in human or animal food, because these residues represent storage forms of possible toxic compounds.[35] In that context, systems such as soybean cell suspension culture are particularly valuable, since they appear to be good models of the non-extractable residue formation in the leaves of dicotyledonous plants.

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